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Surveillance of aquatic animal viruses in seawater and shellfish in Korea

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ABSTRACT

Surveillance of aquatic animal viruses in hosts and seawater is very important to control and prevent the spread of aquatic animal diseases. And also the investigations for shellfish as play role in vector or carrier species, or a candidate host are required. Primer sets for multiplex nested polymerase chain reaction were developed to simultaneously detect aquatic animal viruses (megalocytivirus and white spot syndrome virus as DNA viruses, and viral hemorrhagic septicemia virus, viral nervous necrosis virus [VNNV], and marine birnavirus as RNA viruses) from seawater and shellfish. The multiplex nested PCR amplified each virus specifically with a detection limit of about 10 copies of viral particles and facilitated detection of several aquatic animal viruses from seawater and shellfish. The detection rates of viruses in shellfish samples were not significantly different between areas, except for VNNV which showed much lower positive results in eastern sea area. But, several viruses co-existed in shellfish regardless of the sampling site and time. These results revealed that shellfish might accumulate several viruses over the long-term and could play a role as a bio-indicator or reservoir of viruses derived from aquatic animals.

Statement of relevance

This article is related to field of aquatic animal disease. In this study, we developed multiplex nested PCR assay for detection of aquatic animal viruses from seawater and shellfish. And we found several viruses co-existed in shellfish for long-term, suggesting that shellfish may act as a bio-indicator or reservoir of viruses derived from aquatic animals.

1. Introduction

After an outbreak of an aquatic animal disease, pathogens can be easily released from the host into the environment and re-infect susceptible hosts or be introduced into a carrier or vector species that can retain or transfer the pathogen to other species. In addition, the released pathogens may also accumulate in filter-feeding organisms. Shellfish accumulate human enteric viruses such as norovirus and hepatitis A virus at levels sufficient to cause a disease outbreak in human (Atmar et al., 1993, 1995). Although shellfish cultured near diseased aquafarms can act as carriers or vectors of aquatic animal viruses, studies related to this aspect were not reported fully until recently. Only some studies have examined infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus, marine birnavirus (MABV) and white spot syndrome virus (WSSV) in shellfish and their role as possible vectors or bio-indicators (Gregory et al., 2009; Skar and Mortensen, 2007; Suzuki and Nojima, 1999; Vazquez-Boucard et al., 2010, 2012).

Megalocytiviruses including rock bream iridovirus (RBIV) and flounder iridovirus (FLIV) isolates, viral hemorrhagic septicemia virus (VHSV), and WSSV are annual endemic viral pathogens in Korea and listed as causing notifiable disease by the World Organization for Animal Health. However, information on the carrier or vector species of these viruses is not available. These viruses could be transmitted through seawater and accumulate in shellfish, particularly if shellfish farms are located near diseased fish farms. For this reason, surveillance of aquatic animal virulent viruses with clinical inspection in the host, surrounding seawater, and shellfish is important to control and prevent aquatic animal diseases. To prevent disease transmission, further control measure such as movement limits and disinfection of infected materials are implemented in the event of a disease outbreak. Various agents are generally present in the ambient seawater. In addition, shellfish as filter-feeding organisms accumulate various substances including pathogenic agents from the water. The multiplex polymerase chain reaction (PCR) assay allows various viral pathogens to be detected simultaneously in a single reaction and is very convenient for samples

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containing mixed infected or contaminated substances such as seawater or shellfish.

In this study, we developed a multiplex nested PCR method and investigated several aquatic animal viruses in seawater and shellfish in Korea. We also conducted a genetic analysis of the viruses derived from sea water and shellfish.

2. Materials and methods

2.1. Samples

Seawater and shellfish were collected from different sites in the Eastern (Gijang, Jinha), Southern (Geoje, Gosung, Tongyeong, Wando), and Western Sea (Seosan) areas of Korea (Fig. 1). A total of 245 shellfish samples, clinical healthy on macroscopy, (12 *Chlamys farreri*, 79 *Crassostrea gigas*, 19 *Crassostrea nippona*, two *Haliotis discus hannai*, eight *Meretrix lusoria*, 61 *Mytilus edulis*, two *Panopea japonica*, three *Peronidia venulosa*, 17 *Ruditapes variegatus*, 23 *Saxidomus purpurata*, eight *Scapharca subcrenata*, two *Sinonovacula constricta*, 10 *Tapes philippinarum*, and three *Tresus keenae*) were collected between January 2010 and November 2011. To investigation of aquatic animal viruses in the vicinity of diseased aqua-farms, 64 seawater samples were collected in sterilized 1 L bottles between February 2011 and November 2011 (Fig. 1 and Table 2). All seawater and shellfish samples were directly transported to the laboratory packed in ice within 24 h after sampling.

2.2. Virus

For viral propagation, Chinook salmon embryo (CHSE-214; ATCC CRL-1681), epithelial papilloma of carp (EPC; ATCC CRL-2872), and grunt fin (GF; ATCC CCL-58) cell lines were propagated in minimum essential Eagle's medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibiotic and antimycotic solution (Gibco). Spleens of megalocytivirus (MCV; IVS-1 strain, Jeong et al., 2003)-infected rock bream (90 ± 11.0 g) and brains of viral nervous necrosis virus (VNNV) RGNNV type-infected sea bass were used as viral inoculums for the GF cell line. Kidneys from VHSV- and MABV-infected flounder (10 ± 5 g) were the viral inoculums for the CHSE-214 and EPC cell lines, respectively. Following the development of cytopathic effects (CPEs) showing with 90% on flask, each cell suspension was centrifuged to get rid of the cell debris at 3000 ×*g* for 10 min and the supernatant was stored at −80 °C before the experiment. Fifty milligrams of hepatopancreas of shrimp (*Fenneropenaeus chinensis*) infected with WSSV was used for an experiment.

2.3. Nucleic acid purification from field samples

Shellfish samples were aseptically opened using a knife, the digestive organs were removed, and the digestive gland tissue was separated using a scalpel. The digestive gland tissues of five individuals were pooled as one specimen and stored at −80 °C. The method of Katayama et al. (2002) was modified for the virus concentration from seawater samples. One L of seawater was filtered with a GF/C membrane (1.2 μm pore size, Whatman, Maidstone, UK) and an HA type negatively charged membrane (0.45 μm pore size, Millipore, Tokyo, Japan) to eliminate sediment and absorb the virus, respectively. The cations were rinsed out of the filtered membrane using 100 ml of 0.5 mM H_2SO_4. Then, 10 ml of 1 mM NaOH (pH 10) was passed through the membrane into a new 50 ml tube containing 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of 100× TE buffer for neutralization. The filtrate was concentrated at 3000 ×*g* for 10 min at 4 °C with an ultrafiltration system (Amicon Ultra-15 Centrifugal Filter Unit, 30 kDa, Millipore, MA, USA) and the concentrate was adjusted to a final volume of 1 ml. Nucleic acids were purified from 50 mg of digestive gland tissue and 200 μl of concentrated seawater using an AccuPrep Genomic DNA Extraction kit (Bioneer, Daejeon, Korea) for DNA extraction and an RNeasy Plus mini kit (Qiagen, Valencia, CA, USA) for RNA extraction according to the manufacturer's protocols. The concentration and purity of DNA and RNA was measured by spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany). And the ratio of absorbance between 1.8 and 2.0 at 260/280 nm was assessed as pure for nucleic acids.

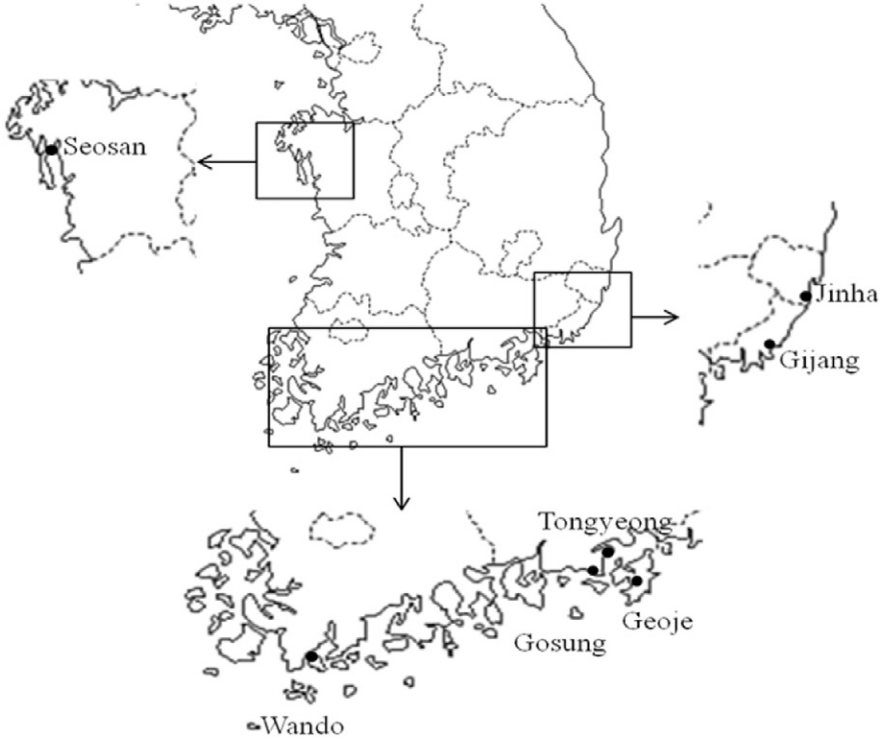

Fig. 1. Sampling sites. Each sample site was located near the seashore in Korea.